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TITLE: Markers of Hypoxia/Reoxygenation in the Development of Metastatic Breast Cancer

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**14. ABSTRACT**

A novel in vitro cell perfusion system was designed and constructed. This system canmaintain cultured breast cancer cells under conditions simulating the hypoxia/reoxygenationcycles observed in vivo. Preliminary studies suggest that breast cancer cells grown underhypoxia undergo oxidative stress as the main cellular antioxidant glutathione is depletedunder these conditions. This suggests that hypoxia and reoxygenation may trigger developmentof the metastatic phenotype and that glutathione may be a marker for the early stages ofprogression.

**15. SUBJECT TERMS**
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## Table of Contents

INTRODUCTION: Cyclical changes in the oxygen content of breast tumor tissue induce extreme oxidative stress. It is this stress that triggers all the complex genetic changes that is characteristic of a metastatic breast cancer cell and enable these cells to survive the migration from the primary tumor, travel *via* the circulatory system and implant in a secondary site. We propose to study the effects of cyclical changes in oxygen content on the metastatic potential of MCF-7 breast cancer cells. In order to do this a novel *in vitro* cell perfusion system must be constructed that can mimic the oxygen fluctuations observed *in vivo*. Cells treated with fluctuating oxygen tension will be compared to cells grown under normoxia and hypoxia for redox balance and metastatic potential. The aim is to discover biochemical and physiological markers of acute hypoxia characteristic of highly metastatic breast cancer.

BODY:

Results are listed relevant to the approved 'Statement of Work'

Task 1. Construct a perfusion system to expose breast cancer cells in 3D culture to periodic episodes of hypoxia and reoxygenation.

An *in vitro* cell perfusion system was designed and constructed and a schematic of this system in shown in Figure 1. It consists of a peristaltic pump that delivers cell culture media from the reservoir to three membrane oxygenators. Two membrane oxygenators are used to equilibrate media with an 20% oxygen/ 5% $CO_2$ mixture. This is equivalent to culture conditions found in most laboratory incubators. The other membrane oxygenator is used to equilibrate media at 1% $O_2$ / 5% $CO_2$. This 1% level of $O_2$ is what we will initially define as 'hypoxia.' Gas delivery is controlled from pre-mixed gas cylinders by two flowmeters (F).

The media from the three oxygenators is delivered to separate solenoidal three-way switches or valves connected to a programmable timer/controller unit. This unit can be programmed to deliver either 20% $O_2$ or 1% $O_2$ to two dishes with cultured cells. A special custom tissue culture dish insert was constructed (Figure 2) that allows flowing media under gas-tight conditions. To test the system, oxygen electrodes (E) were placed in line. These electrodes were connected to a voltmeter (V) interfaced to a PC via an RS232 cable. Electrode readings were recorded using a LabView program written for this system.

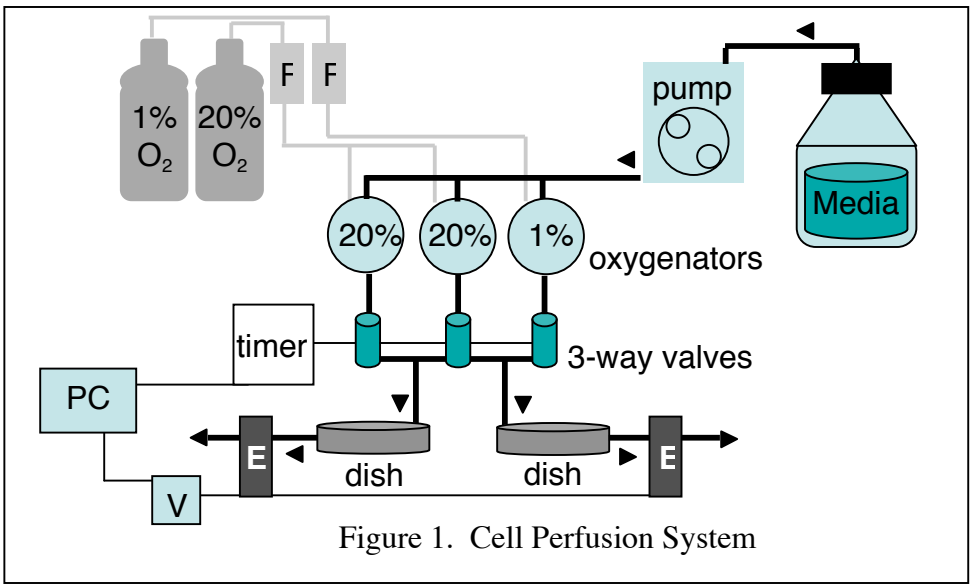


Figure 1. Cell Perfusion System

**Addition to the Original Task 1**: The behavior of cells exposed to the variable oxygen conditions is unknown. Therefore, prior to growing the cells in 3D culture, as stated in the original Task 1, we constructed a system to perfuse cells in monolayer (Figure 2). This allows

visual assessment of cell attachment and viability during the initial exposure to varying oxygen concentrations. This visual monitoring is not possible in 3D culture. This is critical as the perfusion conditions may change cell adhesion and viability which would not be readily detectable in 3D culture. The initial evaluation of this system therefore was done with cells in monolayer culture. The gas tight insert (Figure 2) is designed to fit into a standard tissue culture dish and deliver warmed gas-equilibrated media to cells in monolayer culture. This design has been changed slightly to include multiple inlet and outlet ports to ensure uniform perfusion of the cells in monolayer.

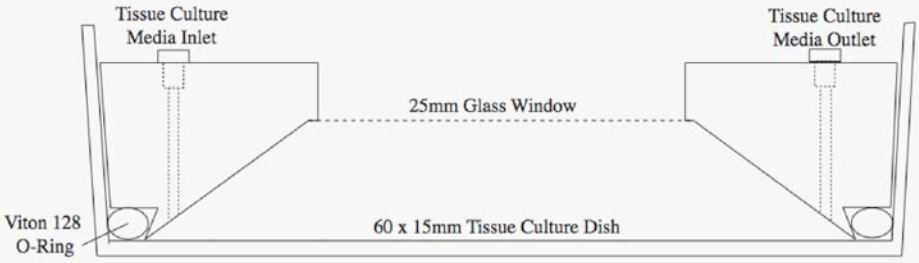



Figure 2. Cell Culture Dish Insert for Perfusion of Cells in Monolayer

The performance of this system for switching between media of difference oxygen concentrations is shown in Figure 3. With only the electrodes in line (no cell culture dishes) the media equilibrated with either 1% or 20% oxygen was switched every 20 minutes. A flow of media at 5 mL/min was used. Trace A in Figure 3 shows the electrode response. Rapid switching is achieved with the lag "L" due to the response time of the electrode. By placing an 80 mm tissue culture dish in line (i.e. similar to that shown in Figure 1), Trace B is obtained. The shape of this curve is influenced by the flow-rate of the media and the mixing that occurs in the volume of the cell culture dish. The arrows show the electrode readings taken from a calibration curve for 20% and 1% oxygen. The absolute electrode readings drift but the difference between the two levels is constant

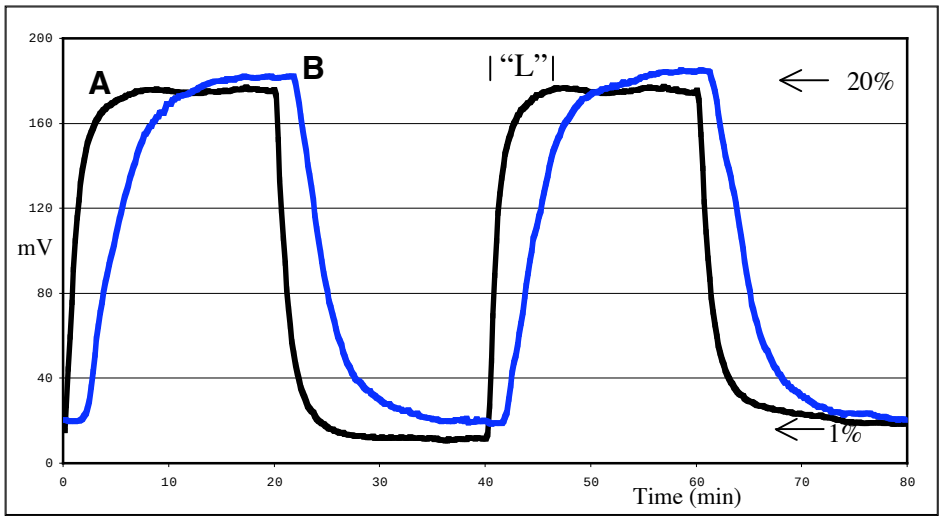



Figure 3. Oxygen Electrode Readings. Trace A, electrode only. Trace B, electrode and tissue culture dish

In addition to dishes exposed to fluctuating oxygen content, a control dish is used to expose cells to flowing media at a constant oxygen content. For example, in the configuration shown in Figure 1, one dish is switched between 1% and 20% $O_2$ whereas the other dish switches between 20% and 20% $O_2$. This is necessary as flowing media can induce shear stress to the cells and can be compensated for in the

control experiments. In all of these experiments, identical cell preparations are placed in an incubator under normoxic conditions as an additional control.

The shape of these curves closely mimics the oxygen electrode readings obtained in vivo in rat mammary tumors [1].

The objective of this task is to design and construct a perfusion system to mimic the in vivo oxygen fluctuations. The results shown in Figure 3 illustrate that we have obtained this objective and the task is complete.

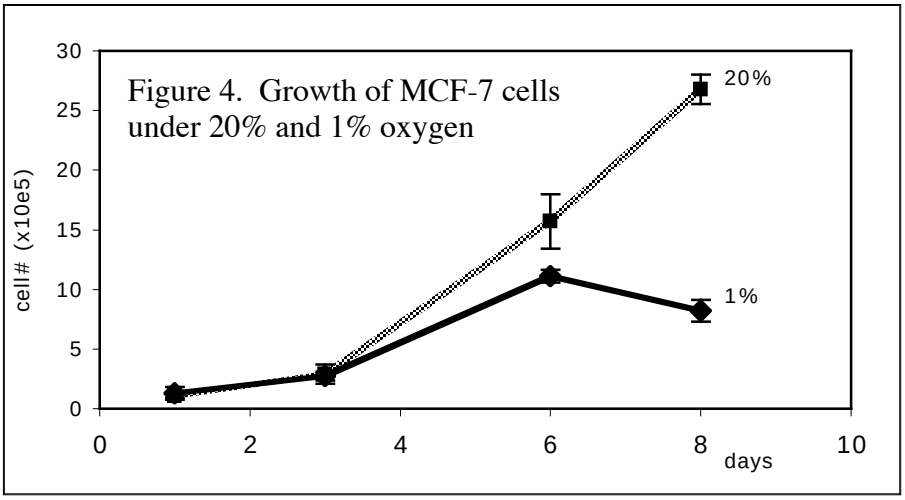
Problems Encountered: This task took much longer to complete than anticipated as some of the customized hardware (particularly the solenoidal switches) were backordered for several months. The original design was modified over the course of this project due to damage to some components. The fragility of these components, i.e. the glass oxygenator, necessitated redesign and currently a system with more robust components is employed in these studies.

Task 2. Use perfusion system to expose MCF7 cells to cyclical periods of hypoxia/reoxygenation (Months 2-12).

Initially, the system has been used to maintain cells under one $O_2$ concentration to follow cell growth and attachment. This cell perfusion system has been used to maintain cells under airtight conditions for up to 7 days. The time limit was mainly due to cells growing beyond confluency during this period. At a flow rate of 5 mL/min, MCF-7 cells did not show any tendency to detach. This is true at both 1% and 20% $O_2$ tension. This is a key point as media flow at this rate does not appear to induce stress due to shear forces. Shear is observed in some bioreactors but at the flow rates used in these studies, does not appear to significantly affect the MCF-7 cells.

In order to compare the effect of cell perfusion on MCF-7 cells, our initial studies are to compare cell growth in cells grown in 20% and 1% oxygen in an incubator and cells grown in the perfusion system as shown in Figure 1. Cell growth curves in monolayer in an incubator under the two oxygen concentrations are shown in Figure 4. The growth under 1% oxygen lags behind that at 20% oxygen. The differences at 6 and 8 days are statistically significant ($P < 0.01$ at 8 days, $P < 0.05$ at 6 days).

Figure 5 shows growth curves for WiDr colon adenocarcinoma cells at 20% oxygen in the incubator (identical conditions as shown in the top trace in Figure 4 for MCF-7 cells, and 1.5% oxygen in the perfusion system. As above for the MCF-7 cells in the incubator, the WiDr cells show slight slower, but we think statistically significant, growth at lower oxygen concentration.



Similarly, an initial study of metabolism (also related to Task 3) we assessed glutathione concentration for cells cultured under 20% and 1% oxygen. Figure 6 shows the glutathione content of cells under both 20% and 1% oxygen. In this case, we compared glutathione levels in both the relatively non-aggressive MCF-7 cells to the highly invasive MDA-MB-231 breast cancer cell lines. In most cases, glutathione levels decrease under lower oxygen. The MDA-MB-231 cells show some different behavior (see day 2) and this is currently being confirmed. This is likely due to increased oxidative stress at lower oxygen and is consistent with some literature reports (e.g. [2]). Other reports however show opposite effects (e.g. [3]). The reasons for these differences will be further probed in future studies.

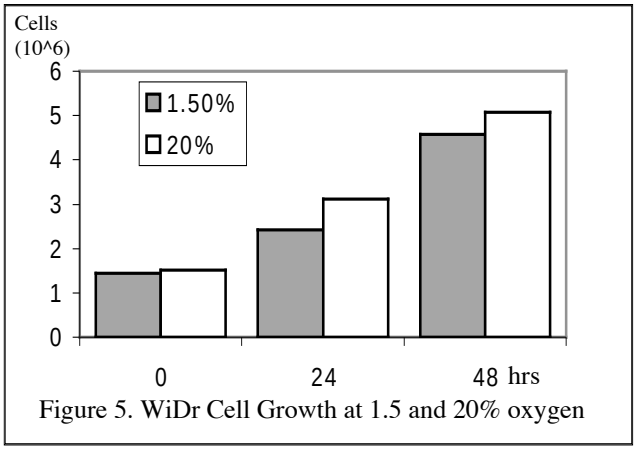


Figure 5. WiDr Cell Growth at 1.5 and 20% oxygen

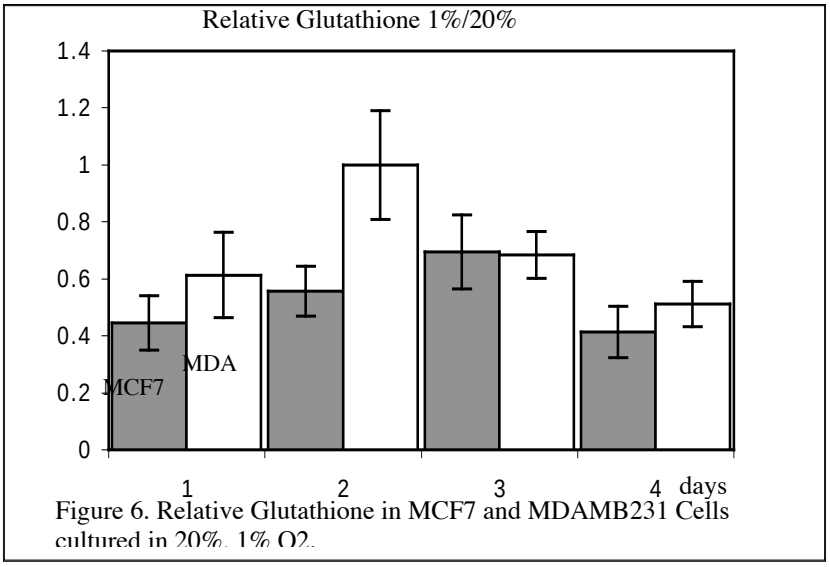


Figure 6. Relative Glutathione in MCF7 and MDAMB231 Cells cultured in 20%, 1% O2.

Problems Encountered: Due to delays in construction of the system as noted above, we have not completed experiments exposing cells to cyclical hypoxia/reoxygenation. These studies are currently underway. Our initial results, presented above provide useful controls for these studies.

Task 3. Collect 'primary' cells in perfusion system, and 'captured' cells for analysis by DNA microarray, proteomic and metabolomic methods (3-12).

We have incorporated a mesh screen (BD-Falcon Cell Strainer, 40 $\mu$ mesh size) at the outflow to our system to capture cells perfused for >24 h at 1% oxygen at a flow-rate of 5 mL/min, No cells were captured in the mesh. The mesh size is likely too large for cell capture and a modified chamber with 10 $\mu$ mesh size is being constructed.

Problems Encountered: The capture of metastatic cells will require a redesign and construction of a vessel that has the capability to capture cells, without damage and introduced little or no backpressure in the perfusion system. This is proving to be more technically challenging than anticipated. However, we have several options to address this problem and are constructing prototypes.

Task 4. Assess metastatic potential of cells expose to different regimens of hypoxia-reoxygenation by transwell-chamber invasion assay (Month 6-12).

We tried coating our own transwell chambers (pore size 8 μ) with Matrigel. The coating thickness was very uneven and we resorted to commercially prepared coated chambers (BiocoatTM)). Using these under normoxic conditions (i.e. 20% oxygen) we obtained the following results with the relatively non invasive MCF-7 line and the highly invasive MDA-MB-231 line (Figure 7). This figure shows the number of cells that penetrated the basement membrane layer and is an in vitro measure of tumor cell metastatic potential. These results confirm that this assay is a good measure of metastatic potential as 50X more MDA-MB-231 cells were counted.

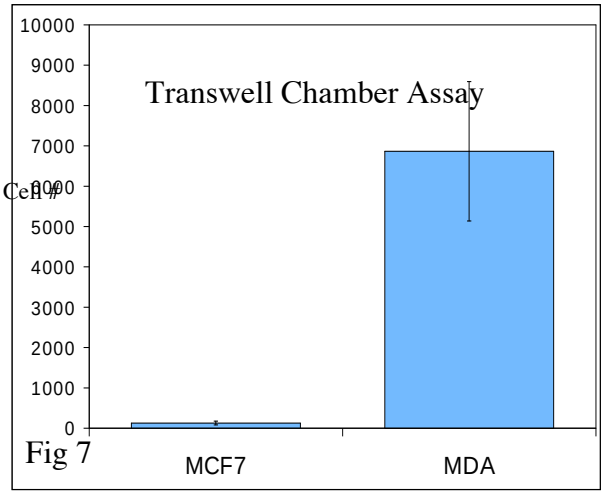


Fig 7

In order to determine if oxidative stress can lead to increased metastasis, we treated MCF-7 cells with 10 doses of hydrogen peroxide ($H_2O_2$) over a period of several months to generate a cell line called MCF-7ox. This cell line, a result of exposure to oxidative stress, was used in the transwell chamber assay. The results are shown in Figure 8. From these data, we conclude that oxidative stress can lead to a more invasive phenotype.

Problems Encountered: The use of commercially produced transwell invasion chambers, while more expensive, has solved problems with reproducibility of membrane coatings. The chambers work well and our preliminary results are as expected. As we continue our work under Task 2, we will be able to assess metastatic potential through these methods.

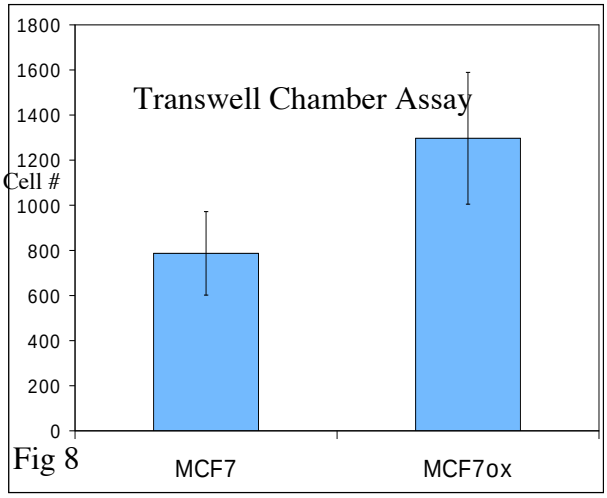


Fig 8

KEY RESEARCH ACCOMPLISHMENTS

1- Novel cell perfusion system allowing cyclical changes in oxygen content was constructed
2- This system mimics cyclical changes in oxygen content observed in human tumors
3- Cell growth of human breast cancer cell lines is reduced under hypoxic conditions
4- Glutathione levels are reduced under hypoxia likely due to oxidative stress
5- Breast cancer cells exposed to factors that induce oxidative stress can lead to a more invasive phenotype.

## REPORTABLE OUTCOMES

Some of this work was presented at the Biomedical Engineering Symposium, NC State University, May 31st, 2007 and the "Tumor Microenvironment" meeting at Duke University, June 26th, 2007. To date, no manuscripts, patents or licenses have been applied for or issued. This work will provide preliminary data for submission of grants to NIH and DoD.

## CONCLUSION

The cell perfusion system constructed through this grant is a unique tool to study oxidative stress induced in breast cancer cells through cyclical changes in oxygen content. These cyclical changes have been observed in both animal models of cancer and in human tumors. Previous to this work, it was difficult to recreate such changes in a controlled *in vitro* model that allows systematic changes in periodicity and concentration. We have not yet fully explored the use of this system in inducing oxidative stress. However, we have shown that oxidative stress in breast cancer cells can lead to a more invasive phenotype and that glutathione provides a biomarker reflecting this stress. These results support our hypothesis that oxidative stress can be used to assess tumor aggressiveness leading to novel ways to diagnose and treat breast cancer.

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## APPENDICES

None

## SUPPORTING DATA

Personnel Receiving Pay from this research Effort:

Michael P. Gamcsik, PhD, Principal Investigator
Stephanie Teeter, BS, Research Technician